Construction of P450-Expressing Tumor Cell Lines Using Retroviruses

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1. Introduction

1.1. Construction of P450-Expressing Tumor Cell Lines

Studies of tumor cell lines expressing individual cytochrome P450 genes are essential for evaluation of the utility of P450 prodrug activation-based cancer gene therapy (1). P450-expressing tumor cells may also be useful to identify novel P450 gene/prodrug combinations (see Chapter 5). The evaluation of candidate P450 genes for use in prodrug activation gene therapy is greatly facilitated by the availability of P450-expressing tumor cell lines, which can be prepared by the retroviral transduction methods described in this chapter. Finally, it should be noted that the methods described in this chapter are not limited to P450 gene transduction, and can readily be applied to the transduction of other genes of interest to cancer gene therapy. In vitro cytotoxicity assays using these cell lines can then be carried out as described in Chapter 7 prior to initiating more costly and labor intensive in vivo tumor studies in animal models (described in Chapter 8).

P450-expressing tumor cell lines are readily established by transduction of tumor cells with a retroviral vector that carries the P450 gene of interest. Retroviruses are excellent vectors for laboratory studies of cancer gene therapy because they can infect dividing tumor cells with high efficiency and may contain nonviral inserts of up to 7.5 kb (cf., full-length P450 cDNA coding sequence ~1.5 kb). These retroviral vectors are prepared in high-titer replication-deficient retrovirus packaging cell lines that express key genes required for retroviral replication (2). Viral titers of ≥10^6 particles per mL of culture supernatant are readily generated, and can be used directly for transduction of
tumor cells in vivo (3), or more commonly, in cell-culture. For cell culture studies, selection with an appropriate drug-resistance marker enzyme encoded by the retrovirus (e.g., puromycin or hygromycin resistance) yields a pool containing thousands of independent, clonal tumor cells, each of which expresses the specific P450 gene of interest (albeit at levels that may differ from one clone to the next). The retroviral long-terminal repeat, which contains the retroviral enhancer and promoter region, ensures stable integration of the P450 gene into the host genome (4).

2. Materials

2.1. Transfection

1. Bosc 23 cells: Ecotropic packaging cell line (5).
2. Growth medium for Bosc 23 cells: Dulbecco’s Modified Eagle Medium (DMEM) (Life Technologies, Gaithersburg, MD, cat. # 12100-046) supplemented with 3.7 g of sodium bicarbonate, 10 mL of penicillin (5000 U/mL), and streptomycin (5000 μg/mL) (Life Technologies, cat. # 15070-063) in 900 mL of deionized water. Adjust the pH of the medium to pH 7.1 with concentrated HCl. Filter-sterilize with 0.2 μm bottle-top filters. Store at 4°C for up to 6 mo. To prepare DMEM containing serum supplement 450 mL of complete DMEM with 50 mL of heat-inactivated (56°C for 30 min) fetal bovine serum (Sigma, St. Louis, MD, cat. # F-2442) (final serum concentration, 10%). Store FBS-DMEM at 4°C for up to 2 mo. Prewarm 10% FBS-DMEM to 37°C before adding to cells.
3. Freezing Medium for Bosc 23 cells: 10% dimethyl sulfoxide and 90% heat-inactivated fetal bovine serum. The medium may be stored at –20°C in 50 mL aliquots for up to 1 yr. Freeze the Bosc 23 cells at 1 × 10⁶ cells/mL and store in liquid nitrogen.
4. 2×HBS (42 mM HEPES, 274 mM NaCl, 10 mM KCl, 1.4 mM Na₂HPO₄, 11 mM glucose), adjusted to pH 6.95 (see Note 1). Filter-sterilize with 0.2 μm bottle-top filters. Store at –20°C in 10 mL aliquots. Stable for 1 yr.
5. 0.25M CaCl₂ (autoclave or filter-sterilize). Store at room temperature or at –20°C.
6. 25 mM Chloroquine (Sigma, cat. # C-6628). Prepare in phosphate-buffered saline or growth medium and filter-sterilize. Store at –20°C in 100 μL aliquots for up to 1 yr.
7. PBS: Phosphate Buffered Saline (without Mg²⁺ or Ca²⁺) 0.146M NaCl, 0.01M NaHPO₄, 2.68 mM KCl, 1.76 mM KH₂PO₄ Filter-sterilize and store at room temperature. The pH will be about 7.4 with the mixture of these salts. Prewarm buffer to 37°C before using.
8. Syringe filters: 0.2 μm 25 mm low-protein binding filter (Gelman, Ann Arbor, MI, cat. # 4454).
9. Syringe filters: 0.45 μm 25 mm low-protein binding filter (Gelman, cat. # 4184).
10. 100 mm tissue culture dishes–Greiner, Solingen, Germany (Intermountain Scientific, Kaysville, UT, cat. # T-2881-2).
2.2. Retroviral Infection and Cell Selection

1. Tumor cell lines: 9L wild-type (6) and 9L/lacZ cell line (β-galactosidase) (ATCC CRL 2200).
2. Polybrene (Hexadimethrine bromide 1,5-dimethyl-1,5, diazaundecamethylene polymethobromide) (Sigma H-9268). Dissolved at 1 mg/mL in DMEM or PBS (see Subheading 2.1., items 2 and 7). Store at –20°C. Stable at 4°C at 1 mg/mL for 1 yr.
3. Retroviral supernatant, frozen aliquot stored at –80°C (prepared as described in Subheading 3.2.).
4. Stock Puromycin (2 mg/mL) (Sigma P-7255) dissolved in PBS (see Subheading 2.1., item 7). Filter-sterilize the puromycin solution using a 0.2 μm syringe filter. Store at 4°C for up to 1 yr.
5. Stock Hygromycin B (50 mg/mL) (Sigma H-0654) (sterile). Store in the dark at 4°C for up to 1 yr.
6. Stock G-418 (1 mg/mL) (Geneticin) (Life Technologies, cat. # 11811-031). Prepare fresh in 10% FBS-DMEM. Geneticin solution is filter-sterilized using a 0.2 μm syringe filter. A solution of 50 mg/ml of active geneticin is stable at 4°C for up to 1 yr. The geneticin stock value is based on the drug activity for cell selection, which is reported by the manufacturer of each product lot.
7. Stock Blasticidin (3 mg/mL) (ICN Pharmaceuticals, Costa Mesa, CA, cat. # 150477). Prepare fresh in deionized, distilled water. Blasticidin solution is filter-sterilized using a 0.2 μm syringe filter. Aliquots of 100 μL can be frozen and stored at –20°C for up to 6 mo.
8. 9L Cell Freezing Medium: DMEM supplemented with 20% FBS and 10% dimethyl sulfoxide. May be stored at –20°C in 50 mL aliquots for up to 1 yr.
9. 1 × Trypsin-EDTA: 10 × Trypsin-EDTA (Life Technologies, cat. # 15400-054) diluted 10-fold in 1 × HBSS (see below). Store at 4°C for up to 6 mo. Prewarm to 37°C before adding to cells.
10. 1 × HBSS: Hank’s Balanced Salt Solution (Life Technologies, cat. # 21250-014) containing 0.35 g of NaHCO₃/L. Filter-sterilize and store at 4°C for up to 1 yr.

2.3. Establishing Individual Clonal Cell Lines

1. 96-well tissue-culture dishes (Greiner–Intermountain Scientific T-3025-1).
2. Cyclophosphamide M.W. 279.1 g/mol (Sigma, cat. # C-0768) potential carcinogen. Prepare fresh for each assay. Dissolve in 10% FBS-DMEM and filter-sterilize using a 0.2 μm syringe filter.

3. Methods

3.1. Method Overview: Construction of P450-Expressing Tumor Cell Lines Using Retroviral Technology

The following is a summary of the steps required for the construction of tumor cell lines that stably express specific cytochrome P450 genes and can be used for preclinical studies of the utility of P450 genes for prodrug activation-based cancer gene therapy. Individual steps are described in greater detail in
**Subheadings 3.2., 3.3., and 3.4.** Several of these steps have been modified or adapted from the protocol described in (5).

1. Subclone the P450 cDNA that is to be expressed into a suitable retroviral plasmid (see Note 2). Expression of the cDNA can be controlled by the retroviral LTR promoter or by an internal promoter (e.g., SV40 or CMV), depending on the retroviral vector chosen.
2. Transform retroviral plasmid DNA into competent bacteria (see Note 3).
3. Select positive clones by growing the transformed bacteria on agar plates containing the appropriate prokaryotic drug marker, e.g., ampicillin or tetracycline.
4. Identify positive bacterial clones by either PCR or restriction enzyme digestion (see Note 4).
5. Sequence the plasmid across the cDNA cloning site to verify the identity of the retroviral plasmid construct (see Note 5).
6. Amplify the retroviral plasmid in bacteria and isolate the plasmid by a maxi preparation (see Note 6).
7. Transfect plasmid DNA into a suitable retrovirus packaging cell line to obtain infectious retroviral supernatant (see Note 7). Following transfection, harvest the supernatant, which contains retrovirus encoding the P450 gene under study (see Subheading 3.2.).
8. Infect tumor cell line with retroviral supernatant and then select a population of retrovirally infected cells with a selection drug appropriate to the retrovirus (e.g., puromycin, in the case of pBabe-puromycin retrovirus) to obtain a pool of retrovirally transduced cells (see Subheading 3.3.).
9. If required, clone out individual cell lines from the heterogeneous pool of retrovirus-infected cells to obtain one or more clonal cell lines with elevated P450 expression levels (see Subheading 3.4.).
10. Characterize pools or clonal cell lines for P450 gene expression by Northern blot, Western blot (7), enzymatic assay and/or immunofluorescence using an anti-P450 antibody.
11. Evaluate the impact of P450 gene expression on the cell’s chemosensitivity to P450-activated prodrugs of interest using the in vitro cytotoxicity assays described elsewhere in this volume (see Chapter 7).

### 3.2. Transfection of Retroviral Plasmid DNA and Harvesting of Retroviral Supernatant

1. Plate $2.5 \times 10^6$ Bosc 23 cells per 60-mm tissue-culture dish approx 24 h prior to transfection. Grow cells in a 37°C humidified incubator containing 5% CO$_2$ (see Note 8).
2. Remove the 10% FBS-DMEM medium and add 4 mL of fresh culture medium containing 25 μM chloroquine (final concentration) 3 h before transfection (see Note 9).
3. Dissolve 24 μg of retrovirus plasmid DNA in 0.5 mL of 0.25M CaCl$_2$ (see Note 10).
4. Add to the CaCl$_2$-DNA solution an equal volume (0.5 mL) of 2 × HBS slowly and evenly, by dropwise addition, over approx 1 min (see Note 11).
5. Remove a small aliquot (about 40 μL) of the calcium phosphate-DNA solution and place on a glass microscope slide. Examine the sample by magnifying the drop with a 40 × objective under a phase-contrast microscope for the presence of small precipitated particles. Typically, a fine precipitate is observed within 1 to 2 min after adding 2 × HBS. At that point, add the remainder of the 1 mL sample of calcium phosphate-DNA solution to the 60-mm tissue-culture dish. Swirl the dish gently to distribute the calcium phosphate-DNA precipitates evenly.

6. Remove the culture medium a minimum of 5 h after DNA transfection, but no longer than 12 h (see Note 12).

7. Wash the cells twice with PBS and replace with 4 mL fresh 10% FBS-DMEM (see Note 13). Place the Bosc 23 cells back in the tissue-culture incubator.

8. Incubate the cells for 24 h to allow for cell recovery, then aspirate and discard the medium (see Note 14). Replace with 4 mL of fresh 10% FBS-DMEM. Place the cells back in the tissue-culture incubator.

9. After a second 24-h period (see Note 15), harvest the cell-culture medium by gently removing the supernatant with a sterile pipet. Transfer the supernatant to a 15 mL polypropylene centrifuge tube.

10. Spin for 5 min at ~ 200 g at 4°C.

11. Filter supernatant through a 0.45 μm low protein binding syringe filter into a sterile 5 mL polypropylene centrifuge tube (see Notes 16 and 17).

12. Store the Bosc 23 cell supernatant containing retrovirus on ice only if the recipient tumor cells are to be infected within ~ 2 h (see Subheading 3.3.). For long-time storage of the retrovirus, snap-freeze the centrifuge tube containing the retroviral supernatant in liquid nitrogen and then place at –70°C (see Note 18).

3.3. Retroviral Infection and Cell Selection

1. Plate sufficient recipient tumor cells to give a confluency of about 50% 24 h later. For 9L gliosarcoma cells and NIH-3T3 cells, plate 0.5 × 10^6 cells/100-mm dish 12 to 18 h prior to infection.

2. For each 100-mm dish of recipient cells, replace the 10% FBS-DMEM cell-culture medium with 3 mL of retroviral supernatant (see Subheading 3.2.) containing 12 μg of filter-sterilized polybrene (final concentration 4 μg/mL) (see Note 19).

3. As a control, incubate a second plate of cells with 3 mL of culture supernatant that is devoid of virus and is prepared by mock-transfection of Bosc 23 cells. These control cells can be used to determine the efficiency of drug selection (i.e., the percentage of cell death in uninfected cells treated with the selection drug).

4. After 3 h, supplement the retroviral supernatant by adding 7 mL of fresh 10% FBS-DMEM to the culture plate. Return the cells to the incubator for an additional 45 h (see Note 20).

5. After a total of 48 h of infection, trypsin digest and divide the near-confluent infected Bosc 23 cells into 4 new 100-mm plates for drug selection. Passage the mock-infected cells at a 1:4 dilution into one new 100-mm plate.

6. After the cells have attached to the tissue-culture plate (between 4 and 16 h), add filter-sterilized antibiotic (e.g., puromycin or hygromycin) to select a population
of retrovirally infected cells that stably express the drug-resistance marker (see Note 21). Add the selection drug to the mock-infected cells as a positive control. The concentration of drug and length of selection time vary with the cell line and with the type of selection drug (see Note 22). For rat 9L gliosarcoma cells, typical conditions for drug selection are as follows:

a. Puromycin: 2 μg/mL for 2 d.
b. Hygromycin: 300 μg/mL for 3 d.
c. G-418: 1.0 mg active G-418/mL for 6 d (replacing medium and G-418 every 2 d up to 6 d).
d. Blasticidin: 3 μg/mL for 2 d.

7. After selection, prepare frozen stocks of the pool of stable cell lines at a concentration of 2 × 10^6 cells/mL of freezing medium and store in liquid nitrogen.

8. Characterize the cell line for P450 protein expression (see Note 23).

9. Verify that the retroviral transduced cell line does not generate and does not contain replication-competent retrovirus (see Note 24).

### 3.4. Establishing Individual Clonal Cell Lines from Heterogeneous Pool of Retrovirally Transduced Cells

In some cases, biochemical analysis of a pool of drug-resistant retrovirally infected cells may reveal a lower than desired overall level of expressed-P450 protein. It may be possible to increase the level of expression of the transduced-P450 gene several-fold by dilution cloning to obtain individual clonal sublines that have a higher level of P450 protein expression than the heterogeneous cell population obtained during the original retroviral infection and drug-selection procedure. These methods are now described.

1. Dilute pool of retroviral transduced cells (see Subheading 3.3.) to calculated concentrations of 0.5, 1, and 4 cells per 200 μL of 10% FBS-DMEM. Distribute the diluted cells into at least one 96-well tissue-culture plate per concentration. Incubate the plate for about 10 d to 2 wk, until single colonies are visible under the microscope in individual wells (see Note 25).

2. Detach the cells growing as single colonies with trypsin-EDTA and divide the colony into two wells of a new 96-well culture plate (see Notes 26 and 27).

3. Allow the cells to attach overnight. When dealing with cells transduced with a prodrug-activating gene, such as a cytochrome P450 gene that activates the prodrug cyclophosphamide, cells in one of the two wells may be treated with the prodrug to identify individual transduced cells that express a higher level of prodrug-activation P450 enzyme.

4. If cyclophosphamide is chosen as the P450 prodrug of interest, add cyclophosphamide (1 mM) to one of the two wells. The paired well remains drug free. Wait 2–3 d, and examine the wells to identify cells that are dying from the drug treatment. When cyclophosphamide-sensitive clones are identified, detach and replate the corresponding untreated cells derived from the same cell colonies into 60-mm dishes.
Fig. 1. Schematic map of the pBabe vector that is used for retroviral transduction to establish stable rodent cell lines expressing P450 or other genes. The retroviral vector contains the retroviral long-terminal repeat (LTR) promoter, a gag gene that lack the initiator methionine start sequence (ATG) and a multiple cloning site (MCS) with unique restriction sites for cloning a P450 cDNA. The mammalian selectable marker encoded by the puromycin resistance gene, in the example shown, is transcribed from the SV40 promoter. The pBabe vector also contains a bacterial selectable marker the ampicillin resistance gene for selection in bacteria (not shown).

5. When the cells are confluent, freeze a portion in liquid nitrogen for cell stocks. Characterize the remainder of cells for P450 protein expression and drug sensitivity.

4. Notes
1. The pH of the 2 × HBS solution is critical for obtaining fine DNA precipitates, which are necessary for efficient transfection.
2. Suitable vectors for retroviral infection include pBabe-based retroviral plasmids with puromycin or hygromycin selectable markers (see Fig. 1) (8,9). Clonetech® sells two retroviral vectors, pLXSN and pLNCX, which transcribe the cDNA insert to be expressed from the viral 3’-LTR or from an internal CMV promoter, respectively. The above two retroviral vectors both confer G-418 resistance to transduced target cells.
   The availability of retrovirus plasmids that encode different drug resistance markers allows for the sequential transduction of tumor cells with several genes that can be selected for individually. In this way, cell lines that encode multiple therapeutic genes, or a P450 gene in combination with the P450 reductase (10) can be obtained. Alternatively, multiple genes linked by internal ribosome entry sequence (IRES) elements can be expressed from a single retrovirus.
3. A standard bacterial transformation protocol can be found in (11).
4. Specific PCR primers, complementary to the retroviral vector and the cDNA insert, respectively, can be used in PCR analysis (12) of bacterial extracts to identify positive clones and to directly establish the orientation of the cDNA insert. Alternatively, restriction digestion (13) of the isolated plasmid DNA can be carried out to identify positive clones and to establish the orientation of the cDNA insert.
5. A standard method for plasmid DNA sequencing is described in (14).
6. A plasmid maxi prep method can be found in reference (15).
7. For ecotropic infection of virus into rodent cells, Bosc 23 is an excellent packaging cell line (2,5). Amphotropic packaging cell lines such as Bing permit viral infection of rodent cells as well as primate cells (5).
8. It is important to use Bosc 23 cells that have not been passaged too frequently (i.e., less than 20 passages). Bosc 23 cells should not be plated too sparsely (≤ 25% confluent) or too densely (≥ 75% confluent). Bosc 23 cell monolayers that appear clumpy rather than uniform may have reduced DNA uptake efficiency.

9. Chloroquine enhances the efficiency of DNA transfection by inhibiting lysosomal formation, thus preventing DNA sequestration and DNA degradation.

10. It is generally not necessary to filter-sterilize the CaCl$_2$-DNA solution if the DNA is prepared using phenol/chloroform extraction methods and if the DNA is dissolved in Tris-EDTA buffer (10 mM Tris-HCl Ph 8.0 1 mM EDTA). If required, filtration of the CaCl$_2$-DNA solution through a 0.2 μm syringe filter can be carried out to ensure that bacteria from the plasmid DNA preparation do not contaminate the Bosc 23 cell culture.

11. Dropping the 2× HBS slowly and evenly is essential for obtaining small calcium phosphate-DNA precipitates. The efficiency with which the cells take up the DNA depends on the size of the precipitate. Large clumpy DNA pellets are too large to enter the cell. Very fine pellets are too light to settle on the surface of the cell and remain suspended in the medium.

12. Chloroquine and the calcium phosphate-DNA transfection precipitates are both toxic to Bosc 23 cells. It is thus essential that they be removed between 5 h (minimum transfection time) and 12 h (maximum transfection time) after addition (5).

13. Wash the Bosc 23 cells very gently. Bosc 23 cells are readily detached from the tissue-culture dish.

14. Place the retroviral supernatant to be discarded in a designated biohazard compartment. Inactivate the infectious virus with a viricidal agent, such as Conflikt (manufactured by Decon Labs and distributed by Fisher Scientific, cat. # 04-35S-52).

15. If the Bosc 23 cells are not yet confluent, it may be useful to wait an additional 24 h before harvesting the supernatant.

16. Use polypropylene rather than polystyrene tubes to avoid cracking the tube when placing it in liquid nitrogen. Store the viral supernatant in 3- to 5-mL aliquots.

17. Spinning and filtering the supernatant (steps 10 and 11) ensures that no Bosc 23 cells remain in the supernatant. Failure to remove all of the Bosc 23 cells could result in mixing of Bosc 23 cells with the recipient cell line and continued generation of fresh infectious virus particles.

18. Do not store viral supernatant in a liquid nitrogen tank that contains tissue-culture cells because the stored cells may become infected by the viral particles. The retroviral supernatant is stable at −70°C. The viral titer decreases if the supernatant is frozen and thawed too often (more than three times).

19. Polybrene is a polycation that facilitates efficient and stable introduction of plasmid DNA into cells (5).

20. The cells are incubated during the first 3 h with a more concentrated retroviral supernatant (i.e., 3 mL of undiluted supernatant) in order to increase the efficiency of viral transduction.

21. The specific drugs that are used to select the retrovirally transduced cells are determined by the drug-resistance marker encoded by the retrovirus. pBabe-based
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retroviral vectors are available with drug-resistance genes selectable with puromycin, hygromycin, geneticin (G418), and blasticidin, among others (8,9).

22. The cell line under study should first be tested with the selection drug to determine the minimum drug concentration that kills ~99% of the uninfected cells within a defined time period (typically ~ 2 to 3 d).

23. P450-expressing cell lines may be characterized for P450 protein expression by Western blotting or specific enzyme assays (7,16).

24. The replication-defective nature of the retrovirus can be verified in the following way. Passage retrovirally infected cells at least three times. Remove the supernatant from the final passage and place on wild-type uninfected cells. Proceed with the infection and drug selection protocol of Subheading 3.3., steps 1–6. Compare the survival of these cells following drug selection to the survival of uninfected cell controls. If replication-competent retrovirus is present, virus amplification will occur resulting in a significant number of recipient cells acquiring drug resistance.

25. Some wells will contain single colonies, whereas others may contain either no cells and or perhaps two or more colonies. Single colonies are readily identifiable by their round shape. An oblong or amorphous shape indicates more than one colony is likely to be present. Wells containing two or more colonies should be discarded. Typically, only 20–40% of the individual wells will contain colonies derived from single cells.

26. If the colonies are large, count the cells in the colony and plate the cells at a density of 1 to 2 × 10^3 cells/well.

27. As an alternative to the drug-sensitivity assay described in Subheading 3.4., steps 3 and 4, half the cells from each single colony can be used to seed two or three wells of a 6-well tissue-culture plate. Once these cells have grown to near confluency, they may be used to prepare total cell extracts for analysis by anti-P450 Western blotting to identify individual clones that express the P450 protein of interest at a high level. The remaining cells derived from the single colony may be grown in several wells of a 6-well tissue-culture dish. Once the cells have grown to near confluency, passage the cells and replate into several 100-mm dishes. Cells may then be grown and prepared for storage in liquid nitrogen.

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References


